PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/00, 16/18, A61K 39/00, G01N 33/574, C12N 9/02

A1

(11) International Publication Number: WO 98/42736

(43) International Publication Date: 1 October 1998 (01.10.98)

(21) International Application Number: PCT/GB98/00878

(22) International Filing Date: 23 March 1998 (23.03.98)

(30) Priority Data:
9705949.7 21 March 1997 (21.03.97) GB

(71) Applicant *(for all designated States except US)*: PROTEOME SCIENCES PLC [GB/GB]; Coveham House, Downside Bridge Road, Cobham, Surrey KT11 3EP (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HOCHSTRASSER, Denis, François [CH/CH]; Chemin de la Savonniere 27, CH–1245 Collonge–Bellerive (CH). REYMOND, Marc, Andre [CH/CH]; Chemin des Coprins 3, CH–1222 Vesenaz (CH).

(74) Agent: LUCAS, Brian, Ronald; Lucas & Co., 135 Westhall Road, Warlingham, Surrey CR6 9HJ (GB).

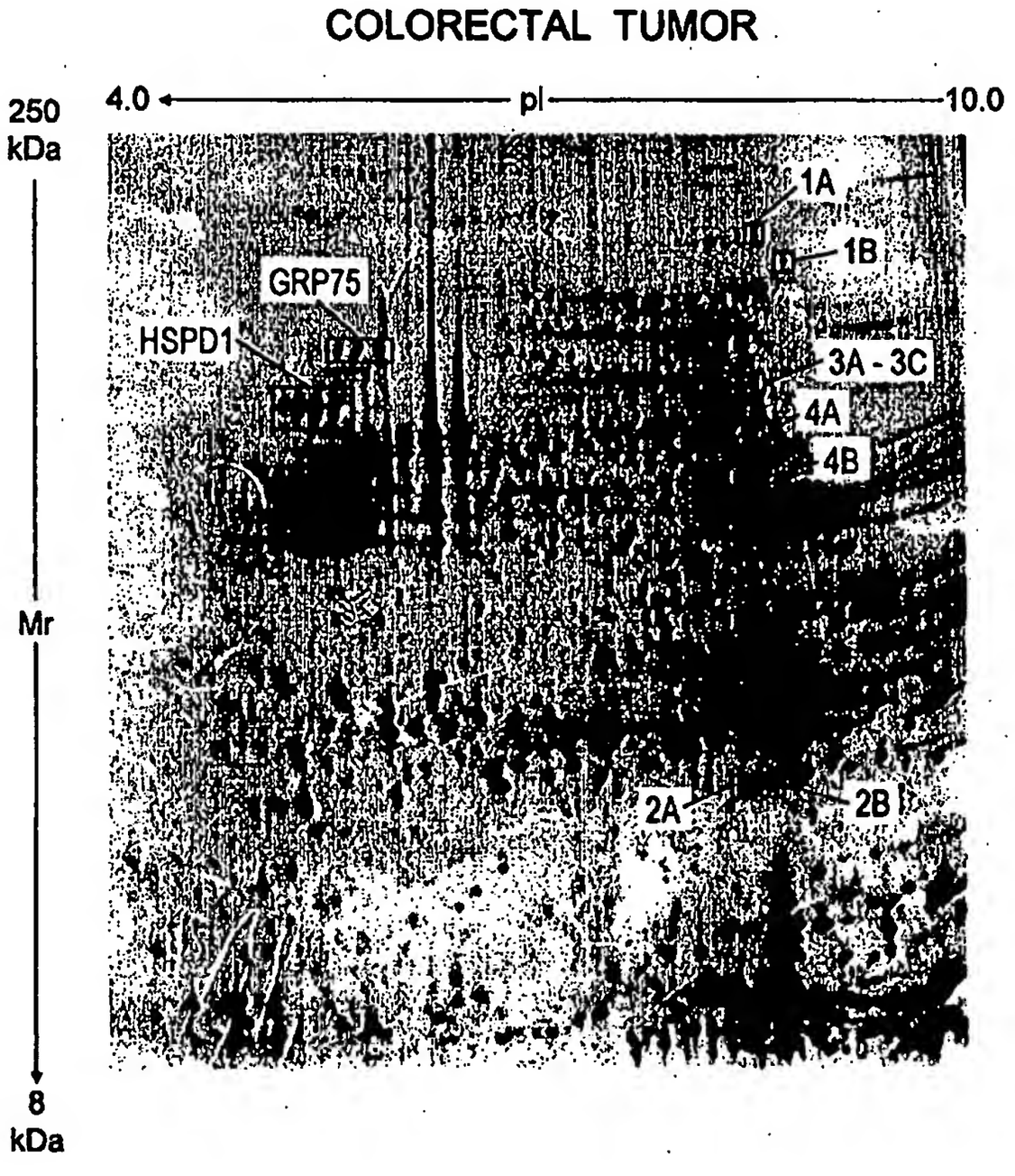
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DIAGNOSIS OF COLORECTAL CANCER AND PROTEINS AND ANTIBODIES FOR USE THEREIN

(57) Abstract

Certain proteins (1A, 1B, 3A, 3B, 3C, 4A, 4B) are present in spots on 2D–PAGE run on protein extracted from epithelial colorectal tumour tissue from patients suffering from colorectal cancer, but are present at a lower intensity or absent in 2D–PAGE run on corresponding normal tissue. These proteins and antibodies thereto are useful in the diagnosis of colorectal cancer.



BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

DIAGNOSIS OF COLORECTAL CANCER AND PROTEINS AND ANTIBODIES FOR USE THEREIN

Background of the invention

1. Field of the invention

This invention relates to the diagnosis of colorectal cancer and proteins and antibodies for use therein.

2. Description of the related art

There is a need for improved methods of diagnosis of colorectal cancer, particularly to determine whether there is any residual disease after surgery or therapy has taken place. This is particularly necessary to detect metastases. The present invention relates particularly to epithelial tumours and other abnormalities of colorectal epithelial cells. The term "colorectal" as used herein refers to the colon or rectum. The term "diagnosis" as used herein includes the obtaining of any kind of information relating to the existence of or condition of a tumour or other abnormality, and includes prognosis.

In clinical practice, the TNM (tumour node metastasis) staging system, "TNM classification of malignant tumours", UICC, Springer, Berlin (1992), still offers the best prognostic information in the case of epithelial tumours, because it describes the extent of tumour involvement at the time of diagnosis. Nevertheless, this system has serious limitations, since some patients with early tumour stages develop metastases after curative surgery, even if the tumour does not reappear locally. Other patients with advanced stages of cancer remain disease-free after the surgery. New diagnostic markers for these tumours are clearly needed.

Particularly in colorectal cancer, there are very few diagnostic markers. In principle, further markers might become available if the genotype or phenotype of the tumour cells could be compared with normal cells.

However, the interpretation of comparative studies on colorectal cancer has so far proved disappointing, because of inter-sample variations and methodological problems. For example, comparison of protein fingerprints obtained from tumour cell lines with those obtained from human colonic crypts proved to have no clinical implications, see Ji *et al.*, Electrophoresis *15*, 391-405 (1994). Another problem is the physiologically high content of proteases in the intestinal mucosa.

Summary of the invention

Two dimensional gel electrophoretic maps have been produced from the epithelial cells of normal and colorectal tumour-bearing patients and compared. This has resulted in the finding that the two protein spots 1A, 1B are over-produced in colorectal tumour cells. These spots are shown in Fig. 2 of the drawings. It is useful to define these spots in these terms, as pI and molecular weights in gel electrophoresis are not absolutely determinable. They can vary according to the method of gel preparation and the presence or absence of other spots. Rather, it is better to view the position of the spots on the map relative to the other spots, in much the same way as stars are identified in the night sky.

The spots 1A, 1B are also shown in Fig. 3 of the drawings, from which it can be seen that three spots 3A, 3B, 3C are also over-expressed in the tumor tissue. These lie just to the right-hand end (increased pI), of the catalase spots present in normal colorectal epithelial cell tissue (see Fig. 1).

The spots 4A, 4B are shown in Fig. 2. 4A is a double spot which is not present in normal tissue, while 4B is a spot over-expressed in tumor tissue.

The spots 1A, 1B, 3A, 3B, 3C, 4A and 4B are herein referred to as "markers" and the proteins thereof as "marker proteins". Individually or in any combination

of two or more, they serve as markers of colorectal cancer. Preferably 1A and 1B are used together as markers.

Figure 2 also shows two spots 2A, 2B, which are less suitable as markers by themselves, but could be used in combination with one or more of the other spots 1A, 1B, 3A, 3B, 3C, 4A or 4B.

The spots are defined by reference to a 2D-PAGE carried out on a protein extract of the sample by a method comprising

(1) centrifuging 1000 microlitres of the protein extract at 10,000 x G at room temperature for 5 minutes to form a pellet, dissolving the pellet in 300 microlitres of a solution containing 8M urea, 40% CHAPS, 40 mM Tris, 65 mM dithioerythritol and a trace of bromophenol blue and loading the resulting sample solution onto 3 mm wide x 180 mm long strips of immobilised pH gradient (IPG) polyacrylamide gel having a non-linear, sigmoidal (S-shaped) pH gradient;

(2) rehydrating the gel overnight with 25 ml of an aqueous solution of 8M urea, 2% w/v CHAPS, 10 mM dithioerythritol and 2% v/v buffers of pH 3.5 to 10 and a trace of bromophenol blue;

(3) loading the sample solution onto the IPG gel strips for first dimension electrophoresis and running the electrophoresis at a voltage which is increased linearly from 300 to 3500 V during 3 hours, then for 3 additional hours at 3500 V, and finally for 17 hours at 5000 V;

(4) treating the IPG gel strips with 100 ml of an aqueous solution containing 50 mM Tris-HCl, pH 6.8, 6M urea, 30% w/v glycerol, 2% w/v SDS, 2.5% v/v iodoacetamide and a trace of bromophenol blue for 5 minutes, to resolubilize the proteins and reduce any -S-S- bonds;

(5) preparing a polyacrylamide gradient slab gel 160 x 200 x 1.5 mm from an aqueous solution containing 9 to

16% acrylamide monomer, 2.6% diacryloylpiperazine cross-linking agent, 5mM sodium thiosulfate, 0.05% tetramethylethylenediamine, 0.1% ammonium persulfate and 0.375M Tris-HCl buffer, pH 8.8, all percentages being w/v, overlayering with sec-butanol for two hours, replacing the sec-butanol with water and standing for 15 hours, overlayering the slab gel with an aqueous solution containing 0.5% agarose, Tris-glycine-SDS at pH 8.3, at 25 mM Tris, 198 mM glycine and 0.1% SDS, heated to 70°C and loading the IPG gel strips thereon for second dimension electrophoresis;

(6) running the second dimension electrophoresis at a constant 40 mA/gel and at 10°C for 5 hours; and

(7) silver staining the gels and scanning them with a laser densitometer, to give a computer-generated image of the stained gel.

Spots 3A, 3B and 3C can be defined without reference to the method of gel preparation as they are very easily identified by their proximity at higher pI, but similar molecular weight, to the catalase spots appearing on a gel from normal cells.

The invention includes the use of a protein indicated on a two dimensional electrophoretic gel by 1A, 1B, 3A, 3B or 3C in the drawings or an antibody thereto and in the diagnosis of tumours. In particular, it includes a method which comprises removing a sample of tissue from the location of a suspected solid epithelial cell tumour, and detecting in the tumour tissue a greater than normal concentration of the protein defined above.

Brief description of the drawings

Fig. 1 is a 2D-gel map of proteins present in normal colorectal epithelial cells of a cell preparation (purified as described below);

Fig. 2 is a 2D-gel map of proteins present in tumorous colorectal epithelial cells of a cell prepara-

tion (purified as described below). The map shows spots 1A, 1B; 3A, 3B, 3C; 4A and 4B.

Fig. 3 is an enlargement of a 2D-gel map similar to that of Fig. 2, showing the spots 1A and 1B and the spots 3A, 3B, 3C.

Fig. 4 is a box plot showing comparative results for 39 patients for spots 1A and 1B. The intensity of the 2D-PAGE spots is compared by plotting a parameter representing relative intensity on the y-axis and representing as a line the intensity values into which 5 to 95% of the samples fell and by a box the intensity values into which 25 to 75% of the samples fell. The line drawn horizontally through the box is the median. Spots from cancerous and normal colorectal epithelial tissue from the same patient were compared.

Fig. 5 is a box plot similar to that of Fig. 4, but relating to results for 6 patients for spots 3A, 3B and 3C. The open polygon and asterisk symbols relate to specific patient samples which did not fall within the main plots.

Description of the preferred embodiments

In the invention, a sample of tissue is removed from the patient by biopsy and the epithelial cells are purified to remove other cells and other tissue to the greatest extent conventionally possible. Preferably the epithelial cells are separated from the surrounding tissue, so that the sample contains at least 90 percent epithelial cells by volume of total tissue of the sample (as determined by fluorescence-activated cell sorting using anti-cytokeratin antibodies, after membrane permeabilization of the cells). This purification of the epithelial cells preferably comprises two steps. In the first step, most of the stroma is separated from the epithelial cells by sizing. That is, the sample can be passed through a 300 micrometre filter, which allows the

epithelial cells to be passed through the mesh, while stroma and larger cells are retained. The epithelial cells are then preferably further separated by filtering them on a filter of smaller size such as 150 to 250, preferably 200, micrometres, effective to enable single cells or small clusters of cells to be collected in the suspension passing through the filter.

The most preferred second step of the procedure comprises reacting the tissue with a monoclonal antibody specific for epithelial cells, collecting the reacted epithelial cells, and subsequently releasing them from the antibody. The monoclonal antibody can be any which reacts with an appropriate epithelial cell surface protein, especially a receptor. A particularly preferred such antibody is Ber-EP4, as described by U. Latza, J. Clin. Pathol. *43*, 213 (1990). This antibody is available in the form of magnetic beads ("Dynabeads") coated with it. Since the magnetic beads can be readily separated magnetically, in the usual way in assay technology, the epithelial cells can be collected magnetically and then washed from the beads.

Another way of separating the cells would be by a non-magnetic heterogeneous method using the same or another ligand such as a monospecific antibody or a peptide which binds strongly to an epithelial cell receptor. The ligand is bound to a solid phase, which is subsequently washed to remove the epithelial cells which have become bound thereto.

The preparation of epithelial cells is then used to determine a phenotypic difference between normal and abnormal cells.

The markers may be detectable as proteins by any of the conventional means, including two dimensional gel-electrophoresis, western blotting with an antibody against the protein, a combination of western blotting

with one dimensional gel-electrophoresis, immunoassay (especially enzyme-linked, enhanced chemiluminescent assay).

A method of assay for presence or amount of the marker in a sample from a patient preferably comprises (consists of or includes) a gel electrophoretic method or immunological method or some combination of the two. Conveniently, the method of testing is wholly or predominantly immunological, i.e. typically it involves interaction between the protein of the invention and an antibody thereto or between an antibody of the invention and another antibody. Immunological methods preferably comprise Enzyme-Linked Immuno-Sorbent Assay (ELISA) or western blotting (also referred to as immunoblotting). They can be carried out either directly on the purified sample (after centrifuging to remove cell wall material etc.) or after making a protein extract.

For immunological assay, antibody is required. To obtain antibody, it is first necessary to purify and identify at least one protein of the marker spots or a protein sufficiently immunologically similar thereto as to have the same specific epitope. The protein may be purified by standard methods. Thus, the spot is excised from the 2D-gel (or electroeluted into solution or electro-transferred to a membrane and excised from the membrane) and, de-stained by a known method.

The purified protein can then be sequenced by any of the well-known methods, including N-terminal sequencing by Edman degradation (or, if the N-terminus of the protein is blocked, applied to a fragment cleaved by CNBr or by digestion with a peptidase). Mass spectrometry may also be used for sequence determination, especially the method of M. Wilm et al., Nature 379, 466-469 (1996), the disclosure of which is herein incorporated by reference. After the protein has been se-

quenced, it can be checked in sequence databases for similarity to other known proteins. If the protein is already known or very similar to one which is already known, an antibody will probably be commercially available. Otherwise, an antibody will have to be raised for the purposes of an immunological method of assay of the marker protein.

The purified protein can be used directly to raise antibodies or a synthetic protein or peptide thereof could be made by recombinant DNA means, using a pool of labelled degenerate oligos as a probe or primer.

With knowledge of the full amino acid sequence of the protein, various peptides thereof or even the full length of protein can be synthesised by the usual methods of peptide synthesis. Peptides thereof can be tested for reaction with the said polyclonal antibodies raised against the full length protein. These peptides which give a reaction can then be synthesised and used in place of the full length protein to raise antibodies or in competition or displacement assays for the protein present in the sample.

The term "antibody", as used herein, includes polyclonal, monoclonal antibodies, fragments of antibodies such as Fab and genetically engineered antibodies. The antibodies may be chimeric or of a single species.

Although the antibodies raised initially may be polyclonal, monoclonal antibodies can be prepared using the well known Köhler-Milstein method. Conveniently, the ascites fluid from mouse-mouse hybridomas is used and screened against the protein purified from spots cut from a preparative gel.

Antibodies may also be raised by expressing the immunoglobulin gene on the surface of a bacteriophage and screening the resultant clones against the specific antigen, i.e. the marker protein of the present inven-

tion. See, e.g., S.L. Morrison in "Molecular Biology and Biotechnology" ed. Robert A Meyers, VCH Publishers Inc. 1995, at page 37, the disclosure of which is herein incorporated by reference.

Protocols for western blotting are well known. After transfer of the protein from the electrophoretic gel, which may be a 1D-gel or a 2D-gel, to a suitable membrane, preferably of nitrocellulose or polvinylidene difluoride, the membrane is blocked to prevent non-specific adsorption of immunological reagents. Typical blocking solutions are of skimmed milk powder or bovine serum albumin. After blocking, the protein can be detected directly or indirectly. Direct detection uses labelled primary antibodies, while in indirect detection a second antibody is raised against the first and the second antibody is labelled. The antibodies are usually labelled with an enzyme such as peroxidase or alkaline phosphatase or with a ligand which has a high affinity for a co-ligand, such as biotin, which has a high affinity for streptavidin and avidin. The protein is then detected by adding an enzyme substrate, conveniently a color-forming one, to read out the enzyme label or an enzyme-labelled co-ligand plus a substrate for the enzyme if a high affinity ligand type of label was used.

An alternative method of quantifying or detecting the presence of the protein is the use of immunoassays, preferably ELISAs, which may be performed on the epithelial cell sample or on protein, isolated or partly isolated by 1D or 2D-gel electrophoresis, transferred to a membrane by blotting. Types of immunoassay useful in this invention include antibody capture assays, antigen capture assays (also called competition or displacement assays) and the two antibody sandwich immunoassay. All the immunoassays require labelled marker protein, antibodies or secondary reagents for detection or quantita-

tion. The labels described above for western blotting may be used. The label may be "read out" or detected by any conventional method, e.g. in an ELISA by using color-forming, fluorescent or chemiluminescent means. Enhanced chemiluminescent assays are particularly preferred and several commercial kits for such assays are known in connection with peroxidase and alkaline phosphatase enzyme labels.

Test kits appropriate to the above and other forms of assay will be apparent to those skilled in the art. Preferred such kits are those for an antibody capture assay and comprise a first an antibody to a protein marker and a labelled second antibody thereto, provided either separately or in the form of a linked double antibody. The test kits may optionally include any of a variety of supports or solid phases, especially plastic tubes and microtiter plates. Procedures for assisting the binding of antigens or antibodies to such supports are well known.

Although heterogeneous assays are cheap and well understood in the art, homogeneous assays may also be used in this invention.

Immuno-PCR may be used as a method of amplifying the signal of an immunoassay described above. In this technique the antibody is covalently linked to a piece of arbitrary DNA comprising PCR primers, whereby the DNA with antibody attached is amplified by the PCR. See Hendrickson *et al.*, Nucl. Acids Res. *23*, 522-529 (1995), the disclosure of which is herein incorporated by reference.

It is, of course possible, to assay the marker protein by a method which comprises at least one dimension of gel electrophoresis. The gel electrophoresis protocols are not to be regarded as limited to those described herein. They may be varied considerably. The

sample is first disrupted, e.g. with a high molar concentration of urea, detergents and dithiothreitol or dithioerythritol to break -S-S- bonds. The first dimension gel is then run in an ampholyte mixture which establishes a pH gradient across the gel, so that the proteins migrate to their isoelectric point, i.e. to a point at which the pH of the gel within the pH gradient established by the ampholytes is equal to the charge on the protein. At this stage, there may be sufficient resolution of the marker spots from others to provide an adequate test for the tissue abnormality. If not, a western blot or immunoassay, using antibody to the protein of the invention, may be performed. Alternatively, the second dimension electrophoresis may then be performed. Here the first dimension gel is loaded, transversely to the direction of current, onto a second gel. This is normally an SDS-PAGE gel, the principle being that charges on the protein are swamped by the effect of the SDS, so that the gel separates the proteins according to molecular weight. This is aided by a higher acrylamide concentration.

The following Example illustrates the invention.

Example

Materials and Methods

Isolation of human colonic crypts

Immediately after human bowel resection surgery for various medical reasons, the surgical specimen was stored on ice. After washing with iced phosphate buffered saline (PBS) solution, a fragment of mucosa (5 x 5cm) was dissected from a healthy portion of tissue, as far removed as possible from the site of disease. The mucosa was then immersed in an ice-cold PBS solution containing EDTA 3mM and a freshly prepared cocktail of proteases inhibitors (leupeptine 50 micrograms/ml, PMSF 0.2 mM and benzamidine 0.8 mM). Crypts were scraped

away from the basal membrane with a scalpel, and then gently pressed through a steel mesh having a pore size of 300 micrometres to separate epithelial cells from stroma. The cell suspension obtained in this way was then filtered through a nylon mesh having a pore size of 200 micrometres. This permitted the isolation of single, or small clusters or epithelial and other cells (lymphocytes, macrophages, etc). The stroma (connective tissue) was not altered by this mechanical procedure and could theoretically be used for further analysis.

Isolation of epithelial cells

After washing and centrifuging the cells at 350 x g for 10 min at 4°C, they were resuspended in the above mentioned solution at a concentration of 2×10^7 cells/ml. This suspension was then incubated at 4°C for 30 minutes with anti-Epithelial Cell "Dynabeads" coated with Ber-EP4 antibodies in accordance with the instructions of the manufacturer (Deutsche Dynal GmbH, Hamburg, Germany). The cells were then collected with a magnetic particle concentrator (MPC-1, Deutsche Dynal GmbH, Hamburg, Germany) and washed five times. A cell count and viability check were performed using trypan blue and showed over 90% viable cells. This first part of the procedure required no more than 90 minutes.

Control

The nature of the cell samples was assessed after labelling the preparation products with fluorescein-conjugated anti-cytokeratin antibodies (CAM 5.2, Perkin-Elmer, Norwalk, Connecticut, USA) for 45 min, in accordance with the instructions of the manufacturer. The samples were then sorted qualitatively with a Fluorescence-Activated Cell Sorter (Epics, Coulter-Immunotech, Hamburg, Germany). It was thus estimated that the sample contained at least 90% epithelial cells by volume of total tissue of the sample at that stage.

Denaturation

After centrifugation, the pellets were denaturated with urea 8M, CHAPS (4% w/v), Tris (40mM) and DTE (65 mM) in accordance with SWISS-2DPAGE protocols, Sanchez *et al.*, Electrophoretics *16*, 1131-1151 (1995), and stored at -20°C.

Analytical 2-D PAGE

Total protein in each sample was assayed as described by the well known Bradford method. 100 micrograms of normal human colonic epithelial cell protein samples were separated by 2-D PAGE. A commercial sigmoidal immobilized pH gradient (IPG) ranging from pH 3.5 to 10.0 (18cm) was used for the first dimension. After equilibration, the IPG gel strips were transferred onto vertical gradient slab gels (9-16% acrylamide) for the second dimension, and run with the Laemmli-SDS-discontinuous system. The crosslinker used was piperazine diacrylyl (PDA 2.6%), and the adjunct catalyst sodium thiosulfate (5mM). A full description of the method is given in the Summary of the Invention above.

Preparative 2-D PAGE

Three mg of normal protein samples were separated by 2-D PAGE and electro-transferred onto PVDF membranes. A commercial immobilized pH gradient (IPG) ranging from pH 3.5 to 10.0 was used for first dimensional separation. In-gel sample rehydration was used, which enabled a higher sample loading, increased resolution and shorter running time. After equilibration, the IPG gel strips were transferred onto vertical gradient slab gels (9-16% acrylamide) for the second dimension. Electroblotting onto PVDF membranes was then effected using a home-made semi-dry apparatus with 10% methanol and 10 mM CAPS as buffer (pH 11) at 200 V for 2 h. The membranes were stained with amido black.

Identification of proteins

Gel matching

After the laser densitometer scanning, 2-D PAGE image analysis was carried out using the MELANIE II software package (Bio-Rad, CA). Protein spots were detected and quantified automatically. The optical density, area and volume were computed and directly related to protein concentration. The relative optical density and relative volume were also calculated to correct for differences in gel loading and staining. The experimental pI and MW of the spots, as well as protein identification, were deduced by the MELANIE II image analysis software (Bio-Rad, Hercules, California, USA) from the liver SWISS-2DPAGE reference map, Sanchez *et al.* (1995) referred to above.

Microsequencing

PVDF membranes were stained with Amido Black, destained with water, and dried. The spots of interest were excised, dried under nitrogen, and kept in Eppendorf tubes at -20°C until microsequencing was performed. Ten to fifteen Edman degradation cycles were performed for each spot and the SWISS-PROT database, see Bairoch *et al.*, Nucleic Acids Res. 22, 3578-3580 (1994) was searched to establish the identity of already known proteins.

Immunoblotting

After electrophoretic transfer onto PVDF membranes, the latter were blocked with 10 mM Tris-HCl (pH 7.0), 500 mM NaCl, 0.05% Tween-20 and 0.5% non-fat dry milk prior to incubation with the primary antibodies. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies were used at a 1/1000 dilution as secondary antibodies. Blots were developed using enhanced chemiluminescence and X-ray film as described by Boehringer Mannheim. A monoclonal antibody anti-TCTP was used as an internal marker for control and comparison

with reference maps.

(5) Results

(a) Gel Preparation

The above-described size-separation and magnetic separation using "Ber-EP4" coated "Dynabeads" permitted enrichment of the samples to over 95% epithelial cells. This pure state was assessed by a second consecutive staining using fluorescein-conjugated anti-cytokeratin antibodies followed by Fluorescence Activated Cell Sorting. A quality control using scanning electron microscopy showed no degradation of the cell surfaces. Contamination by blood, as assessed by serum albumin, was low after washing and enrichment. Comparison of samples before and after separation with the "Dynabeads" showed an improvement in contrast and disappearance of degradation patterns. A mean number of 900-1200 spots/gel was obtained using silver staining.

(b) Normal Colonic Epithelium

Using the above methods, a 2D-PAGE map was made from protein extracted from normal colorectal epithelial cells. 40 proteins were identified and marked on a map, shown in Figure 1. This map was published during the priority year in Reymond *et al*, Electrophoresis *18*, 2842-2848 (1997), which also lists the apparent pI and molecular weight of the spots. Only those with high pI and/or molecular weight are relevant to the present invention. These are shown in the Table below:

Table (Key to Figure 1)

Short Name	Protein Description	Apparent pI	Apparent Mol. wt. (kD)
EGFR	Epidermal growth factor receptor	5.75	141,900
GRP75	Mitochondrial stress-70 protein precursor	5.40	73,173
GRP75	Mitochondrial stress-70 protein precursor	5.32	73,173
CAT	Catalase	6.50	57,735
CAT	Catalase	6.74	57,735
CAT	Catalase	7.07	57,615
GLUD1	Glutamate dehydrogenase 1 precursor	7.26	52,813
GLUD1	Glutamate dehydrogenase 1 precursor	6.50	52,813
GLUD1	Glutamate dehydrogenase 1 precursor	6.91	52,703
PMS2	DNA mismatch repair protein	6.39	51,925
PAI-1	Plasminogen activator inhibitor-1	6.50	46,534
SOD-2	Superoxide dismutase (Mn)	7.36	21,182
SOD-2	Superoxide dismutase (Mn)	7.78	21,182

(c) Colorectal Tumour Epithelium

This work was repeated using fragments about 1cm^3 from a colorectal tumour, cut into small pieces, in place of the mucosal crypts. 2D gel maps such as shown in Figure 2 were obtained. Referring to Figures 2 and 3, spots 1A and 1B have a mw of around 80-85kDal and appear in the tumour protein map of Figure 2, but not in Figure 1. Spots 2A, 2B, 3A, 3B and 3C appear at a

greater intensity in the tumour cell map of Figure 2 than in the normal cell map of Figure 1.

Spots 1A, 1B, 3A, 3B and 3C, 4A and 4B and the proteins thereof are useful as markers in the diagnosis of colorectal cancer, whether from samples prepared from epithelial cells or otherwise. Spots 2A and 2B and the proteins thereof can be used in conjuction with any of these, especially 1A and/or 1B.

Spot 1A has a pI of about 7.65 and a molecular weight on this gel of around 80-85kD. However, such a high molecular weight is not at all accurately measurable by gel electrophoresis. It was identified by mass spectroscopy as a polymeric form of manganese superoxide dismutase (Mn SOD). The protein was cut from preparative gels, digested in trypsin and blotted onto a PVDF membrane. The MALDI-TOF method was used to determine very accurately the molecular weights of fragments. MALDI-TOF=Matrix-Assisted Laser Desorption Ionization mass spectrometry, with Time of Flight determination. See P. Jungblut *et al.*, Electrophoresis 17, 839-847 (1996), the disclosure of which in relation to MALDI is herein incorporated by reference. The time taken for various fragments to pass through the magnetic field is measured as Time of Flight. This method is so accurate that molecular weights can be matched to combinations of amino acids. The possible sequences of the fragments are then matched against a database of proteins. The MALDI-TOF mass spectrum for spot 1A showed 5 peaks out of 8, at m/z=1028.76, 1090.66, 1424.94, 1739.07 and 2542.57, which matched sequences of the human MnSOD precursor (amino acid numbering 115 to 123, 124 to 132, 76 to 89, 54 to 68 and 54 to 75). The human MnSOD precursor protein has 222 amino acids and a molecular weight of 24,700. From this result and considering the molecular weight of spot 1A on the gel, it has been

characterised as an approximately tetrameric MnSOD. This result was confirmed by MSMS mass spectroscopy.

Spot 1B has a pI of about 8.0 and a lower molecular weight than 1A. It comprises an approximately tetrameric MnSOD and other polypeptide(s). This spot will require further separation into its components before monoclonal antibodies are raised against it.

Spots 2A and 2B are probably variant forms ("isoforms") of monomeric superoxide dismutase (molecular weight about 21,000 on the gel). Such variants frequently arise in 2D-PAGE, because of different degrees of glycosylation (affecting the molecular weight) or mutations of one or two amino acids (affecting the charge on the molecule and therefore pI).

Spots 3A, 3B and 3C, best seen in Figure 3, have pI about 7.3, 7.5 and 7.7, molecular weight about 56,000-57,000 on the gel. These are believed to be variant forms of catalase, which is one of the proteins identified in Figure 1.

Spots 4A and 4B, Figure 2, have pI about 6.8 and 7.6 and molecular weights about 45,000-46,000 on the gel. 4A is a double spot close to plasminogen-activator inhibitor-1 (PAI-1 in Figure 1). The proteins within the double spot can be resolved and purified. 4B is a spot of about the same molecular weight as 4A over-expressed in the tumour tissue compared with normal.

(d) Analysis of samples from patients

The following data relates to 39 patients (25 men, 14 women) presenting with colorectal cancer. They were aged 57.5 to 65.1 years. 37 were diagnosed by conventional means as having adenocarcinomas, while 2 as having familial adenomatous polyposis. The localisation of the tumours was as follows: rectum = 11; left colon = 11; right colon = 13; small bowel = 1; metastasis to liver = 3. The stage of the cancer was defined in terms

of the UICC (Union International Contre le Cancer) stages as described in UICC: TNM classification, 5th edition, Wiley, New York (1997). The numbers of patients at each stage were: Stage I = 5; II = 14; III = 9; IV = 11.

Samples of normal and tumour tissue were taken from the same patient in as near as possible the same area of the organ affected. The epithelial cells of the sample were purified and 2D-PAGE run as described. Spots 1A and 1B were used (separately) to diagnose the cancer. It was found that these spots gave a positive result in 95% of cases, while in only one case was a false positive given. The results are depicted by the box plot of Figure 4. Referring to Figure 4, the vertical axis represents intensity of the gel spots expressed as a percentage by volume of total darkness. In other words, it is a relative measure of intensity, valid for comparing the normal with the cancerous tissue. The plots "normal" and "cancer" are side-by-side comparisons for the spots from the respective tissues. The thin vertical line represents the intensities of from 5% (bottom) to 95% (top) of the spots, the box from 25 to 75% and the line across the box the median (50%). There was just one normal sample which gave an intensity as high as 5%, as represented by the slight emboldening of the bottom horizontal axis. Thus, each of the spots 1A and 1B gave a very clear distinction between positives and negatives.

Figure 5 shows a similar box plot, but for 6 patient samples only, relating to spots 3A, 3B and 3C.

CLAIMS

1. A protein present in a 2D-PAGE gel spot 1A, 1B, 3A, 3B, 3C, 4A or 4B shown in Figure 2 or Figure 3 of the accompanying drawings, said protein being present in epithelial cells of a tumour of the human colon or rectum in a greater concentration than in normal cells of the same location in the human colon or rectum.

2. A protein according to Claim 1, wherein the spot is 1A or 1B.

3. A protein according to Claim 1 or 2, wherein the 2D-PAGE gel has been prepared by extracting protein from purified epithelial cells of a human patient suspected of having colorectal cancer, followed by:

(1) centrifuging 1000 microlitres of the protein extract at 10,000 G at room temperature for 5 minutes to form a pellet, dissolving the pellet in 300 microlitres of a solution containing 8M urea, 40% CHAPS, 40 mM Tris, 65 mM dithioerythritol and a trace of bromophenol blue and loading the resulting sample solution onto 3mm wide x 180mm long strips of immobilised pH gradient (IPG) polyacrylamide gel having a non-linear, sigmoidal (S-shaped) pH gradient;

(2) rehydrating the gel overnight with 25ml of an aqueous solution of 8M urea, 2% w/v CHAPS, 10mM di-thioerythritol and 2% v/v buffers of pH 3.5 to 10 and a trace of bromophenol blue;

(3) loading the sample solution onto the IPG gel strips for first dimension electrophoresis and running the electrophoresis at a voltage which is increased linearly from 300 to 3500 V during 3 hours, then for 3 additional hours at 3500 V, and finally for 17 hours at 5000 V;

(4) treating the IPG gel strips with 100ml of an aqueous solution containing 50 mM Tris-HCl, pH 6.8, 6M urea, 30% w/v glycerol, 2% w/v SDS, 2.5% v/v iodaceta-

mide and a trace of bromophenol blue for 5 minutes, to resolubilize the proteins and reduce any -S-S- bonds;

(5) preparing a polyacrylamide gradient slab gel 160 x 200 x 1.5mm from an aqueous solution containing 9 to 16% acrylamide monomer, 2.6% diacryloylpiperazine cross-linking agent, 5mM sodium thiosulfate, 0.05% tetramethylethylenediamine, 0.1% amonium persulfate and 0.375M Tris-HCl buffer, pH 8.8, all percentages being w/v, overlayering with sec-butanol for two hours, replacing the sec-butanol with water and standing for 15 hours, overlayering the slab gel with an aqueous solution containing 0.5% agarose, Tris-glycine-SDS at pH 8.3, at 25 mM Tris, 198 mM glycine and 0.1% SDS, heated to 70°C and loading the IPG gel strips thereon for second dimension electrophoresis;

(6) running the second dimension electrophoresis at a constant 40 mA/gel and at 10°C for 5 hours; and

(7) silver staining the gels and scanning them with a laser densitometer, to give a computer-generated image of the stained gel.

4. Antibody to a protein claimed in Claim 1, 2 or 3.

5. Antibody according to Claim 4 which is monoclonal.

6. A method of diagnosis of colorectal cancer, which comprises removing a sample of tissue from the location of a suspected solid epithelial cell tumour, and detecting in the tumour tissue a greater than normal concentration of the protein defined in Claim 1, 2 or 3.

7. A method of diagnosis of colorectal tumours, which comprises removing a sample of tissue from the location of a suspected solid epithelial cell tumour, and detecting in the tumour tissue a greater concentration of the protein defined in Claim 2 than is present in corresponding normal cells.

8. A method according to Claim 7 wherein the diagnosis comprises carrying out an immunoassay in which

the epithelial cells are purified and are reacted with a labelled antibody claimed in Claim 4 or 5, and the label is detected.

9. Use of a protein present in a 2D-PAGE gel spot 1A, 1B, 3A, 3B, 3C, 4A or 4B shown in Figure 2 or Figure 3 of the accompanying drawings, said protein being present in epithelial cells of a tumour of the human colon or rectum in a greater concentration than in normal cells thereof, or an antibody thereto, in the diagnosis of tumours.

10. Use of a protein present in a 2D-PAGE gel spot 1A, or 1B shown in Figure 2 or Figure 3 of the accompanying drawings, said protein being present in epithelial cells of a tumour of the human colon or rectum in a greater concentration than in normal cells thereof, or an antibody thereto, in the diagnosis of tumours.

FIG. 1

COLORECTAL EPITHELIA CELL 2-D PAGE REFERENCE MAP

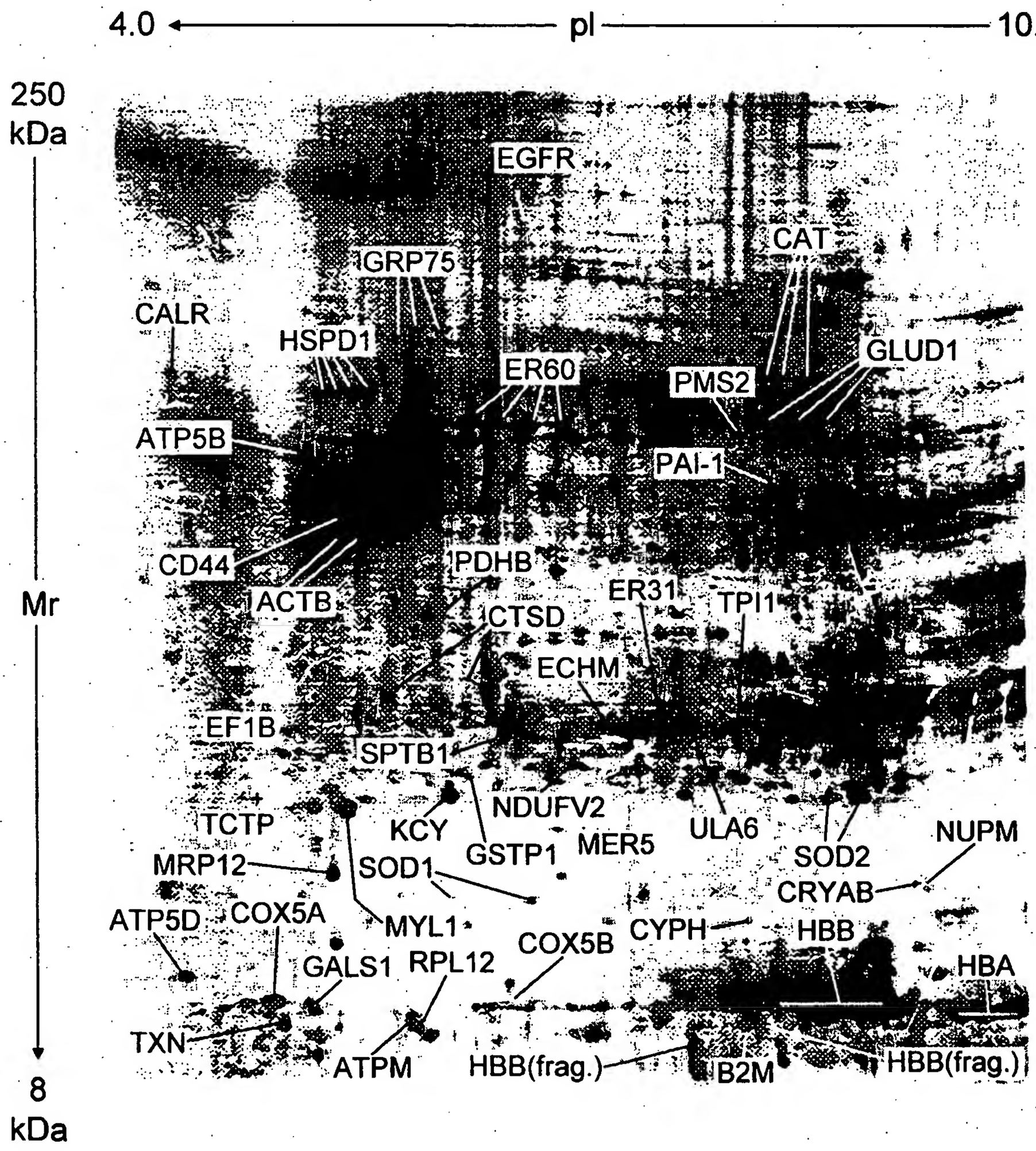
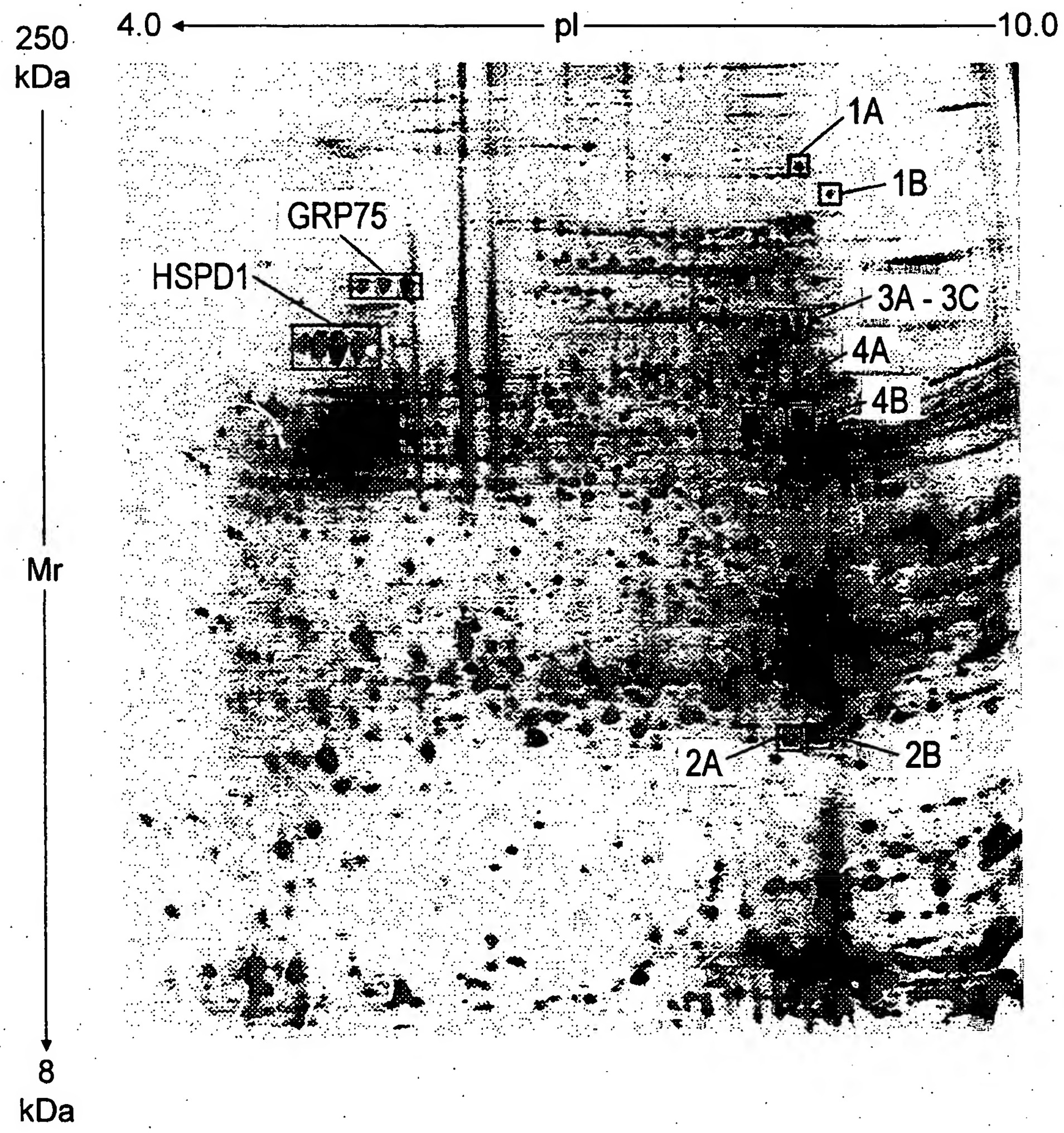


FIG. 2


COLORECTAL TUMOR
250
kDa
4.0
pI
10.0
1A
1B
GRP75
HSPD1
3A - 3C
4A
4B
2A
2B
Mr
8
kDa

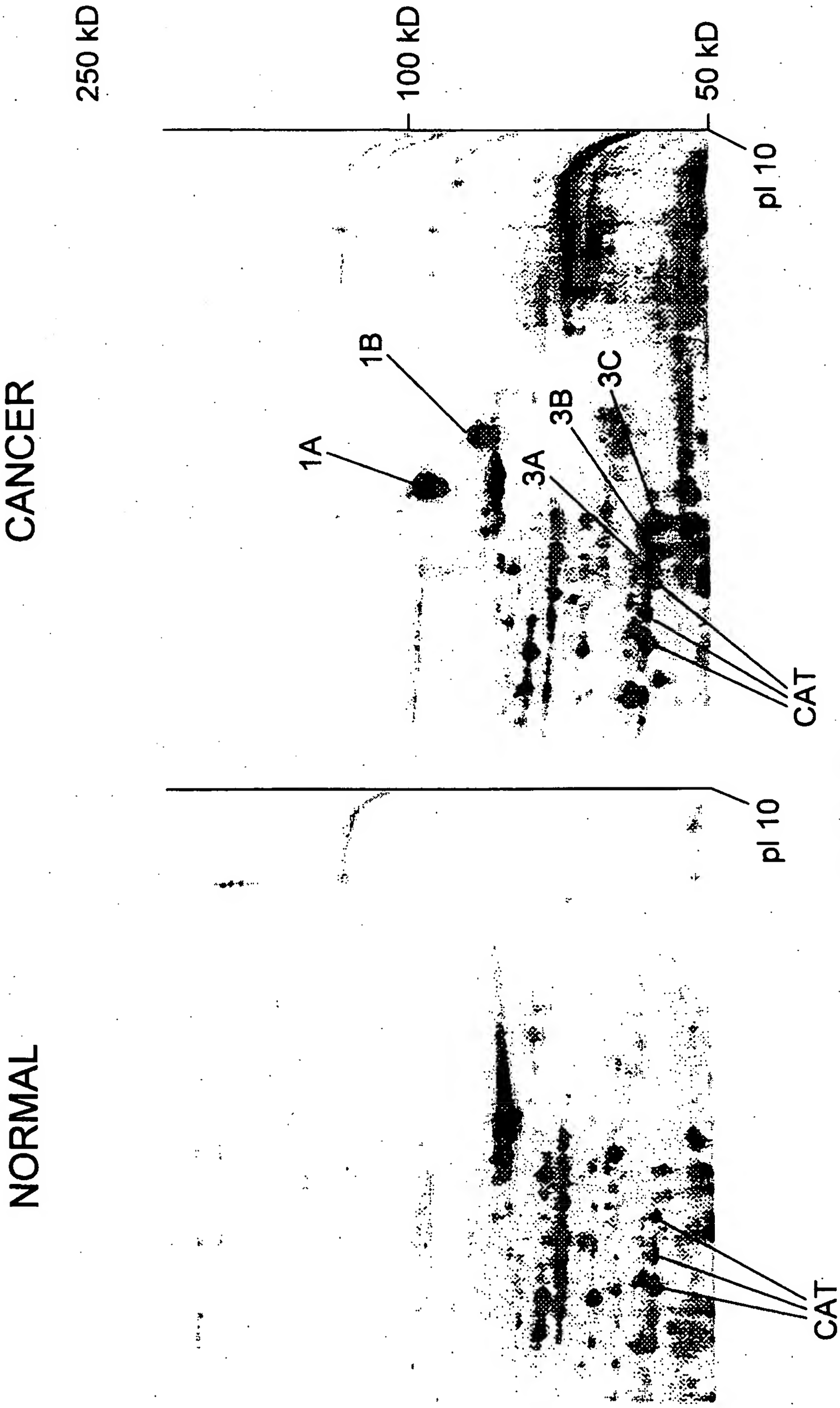

250 kD
100 kD
50 kD
pI 10
CANCER
1A
1B
3A
3B
3C
CAT
pI 10
NORMAL
CAT

FIG.3

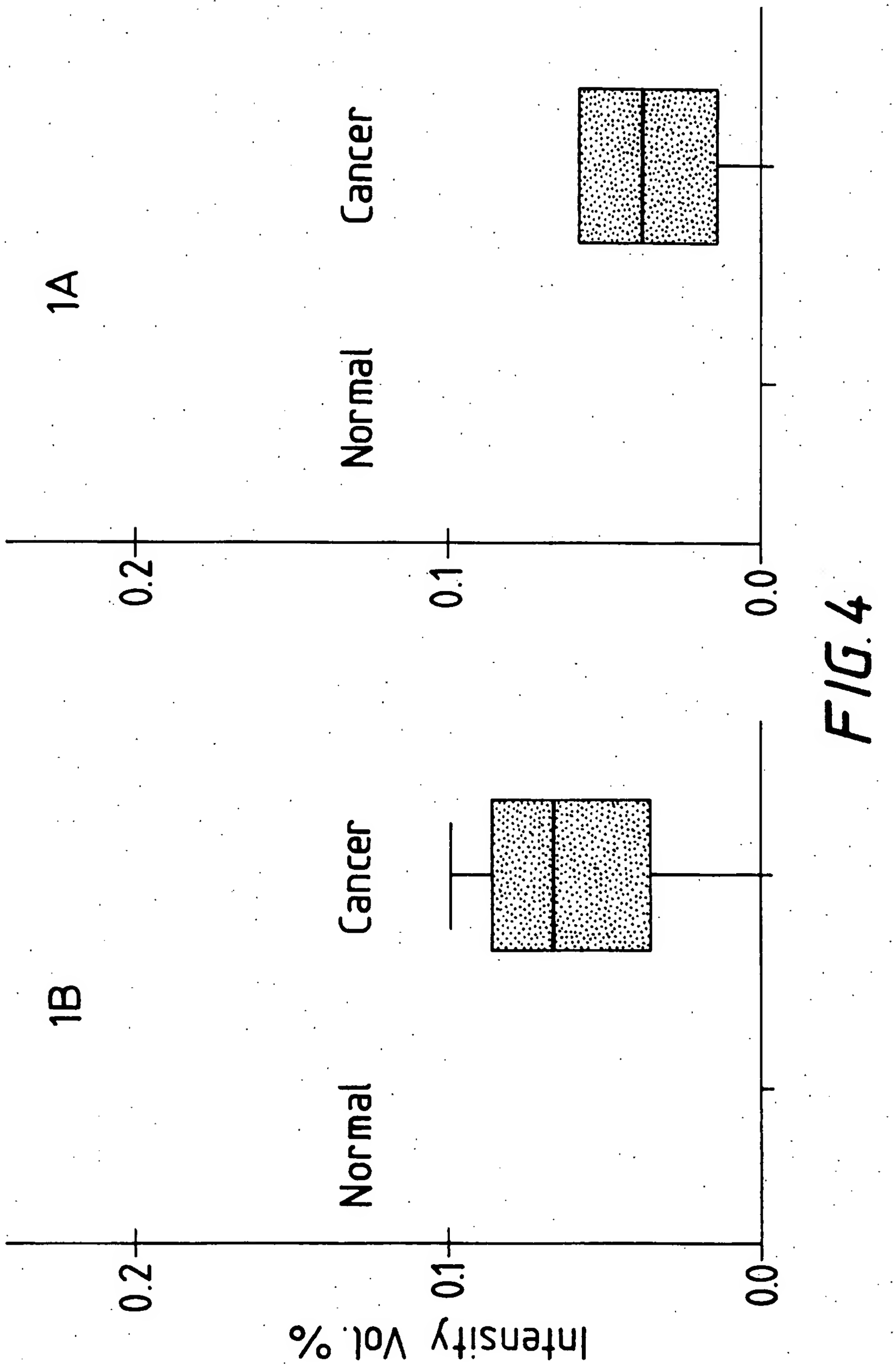
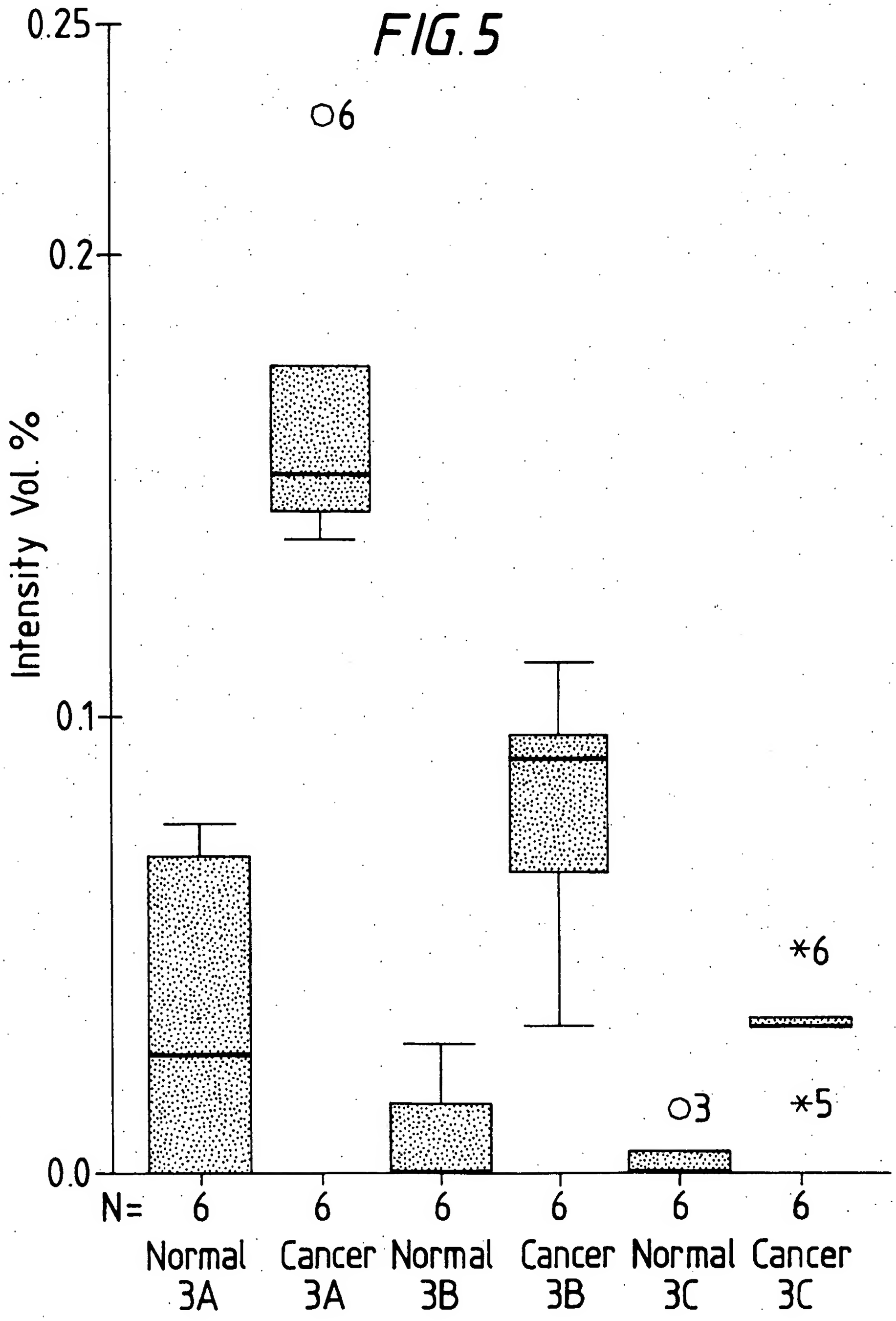

1A
Cancer
Normal
0.2
0.1
0.0
1B
Cancer
Normal
0.2
0.1
0.0
Intensity Vol. %

FIG. 4

5/5

FIG. 5


0.25
0.2
0.1
0.0
Intensity Vol. %
○6
*6
○3
*5
N= 6 6 6 6 6 6
Normal 3A
Cancer 3A
Normal 3B
Cancer 3B
Normal 3C
Cancer 3C

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 98/00878

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/00 C07K16/18 A61K39/00 G01N33/574 C12N9/02

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	REYMOND ET AL: "Standardized characterization of gene expression in human colerectal epithelium by two-dimensional electrophoresis" ELECTROPHORESIS, vol. 18, no. 15, December 1997, pages 2842-2848, XP002071492 see the whole document ---	1-10
X	WO 95 16919 A (MATRITECH INC) 22 June 1995 see the whole document ---	1-10
X	EP 0 349 113 A (UBE INDUSTRIES) 3 January 1990 see the whole document ---	1-10

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 1998	28/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 98/00878

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,O	H. JI ET AL.: "A two-dimensional gel database of human colon carcinoma proteins" ELECTROPHORESIS, vol. 18, no. 3-4, 16 - 18 September 1996, pages 605-613, XP002071493 see the whole document ---	1-3,6,7, 9,10
X,O	M. REYMOND ET AL: "Specific sample preparation in colorectal cancer" ELECTROPHORESIS, vol. 15, no. 3-4, 16 - 18 September 1996, pages 622-624, XP002071494 see the whole document ---	3
X	SALVATORE M ET AL: "DETECTION OF COLON CANCER WITH RADIOLABELLED MONOCLONAL ANTIBODIES" INTERNATIONAL JOURNAL OF RADIATION APPLICATIONS AND INSTRUMENTATION PART B: NUCLEAR MEDICINE AND BIOLOGY, vol. 16, no. 2, 1989, page 103/104 XP000008198 see the whole document -----	4-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/00878

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 9 and 10 are directed at least in part to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte. ional Application No
PCT/GB 98/00878

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9516919 A	22-06-1995	US 5547928 A	20-08-1996
		AU 1437895 A	03-07-1995
EP 0349113 A	03-01-1990	JP 2231568 A	13-09-1990
		JP 2232562 A	14-09-1990
		JP 1299300 A	04-12-1989
		JP 2567664 B	25-12-1996
		JP 2025750 A	29-01-1990
		DE 68921374 D	06-04-1995
		DE 68921374 T	05-10-1995
		US 5147783 A	15-09-1992
		CN 1040821 A	28-03-1990

This Page is inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☒ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURED OR ILLEGIBLE TEXT OR DRAWING
- ☒ SKEWED/SLANTED IMAGES
- ☐ COLORED OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning documents *will not* correct images problems checked, please do not report the problems to the IFW Image Problem Mailbox